3. Experimental Design:

- Collection of Denim textile wastewater
- Isolation of indigenous adapted bacterial strains (Plate assay method)
- Screening of selected adapted bacterial strains (Broth dilution assay method)
- Identification of selected bacterial strains
  - Through 16S rRNA sequence analysis
  - Based on their Biochemical and Morphological techniques according to Bergey’s manual of systemic bacteriology
- Different treatment trials on Denim industrial wastewater by using:
  - Free bacterial pure broth cultures
  - Ca-alginate immobilized cells
  - Biofilm development on inert PUF cells
- Treatment trial on Crude Indigo dye decolorization by mixed adapted bacterial supernatant
- Optimization of different cultural conditions for Indigo dye decolorization efficiency
  - Using, (Khlifi et al., 2010)
    1. Effect of Retention time (8, 16, 24, 32, 40, 48, 56, 64 & 72 h).
    2. Effect of initial pH (5, 6, 7, 8, 9).
    4. Effect of initial organic loading rate (20%, 40%, 60%, 80% and 100%).
    5. Effect of biofilm optimization
- Optimization of different cultural conditions
- Characterization of secondary metabolites using various analytical methods:
  - GC-MS; FTIR; LCMS; H^+NMR; TLC; SEM
  - Cytotoxicity assay
- Characterization of the degradation efficiency of textile wastes through Adsorption isotherm kinetics
- Mathematical modeling on bioremediation efficiency of multiple species used as Biobarrier model.
- Purification of *P. aeruginosa ThrH* protein (Wang and Ng, 2004).
  - Ammonium precipitation; Dialysis; DEAE-Sepharose A-50 column chromatography
  - SDS-PAGE; N- Terminal Sequence analysis
- Molecular Docking studies – Interaction of 3D structure of Indigo dye with various receptors on ‘ThrH’ protein (Gusakov et al., 2000)

Treatment trials carried out Industrial scale
3.1. Sample Collection

Textile effluent sample was collected from the outlet of denim industry dyeing unit in Perundurai KG Fabrics (Tamil Nadu, India) contaminated with the crude indigo dye. The effluent was collected from wastewater outlet and carried in different 10 liters sterile plastic canes and stored in cold condition until use. This sample was used for both isolating indigenous microorganisms as well as for treatment process.

3.2. Reagents and Chemicals

All reagents and chemicals including indigo blue were analytical grade and purchased from sigma Aldrich. The synthetic liquid Indigo dye (Indigotin) is 80% pure with a chemical formula of C₁₂H₈O₂N₂. The naturally occurring substance is indican, which is colorless and soluble in water. Indican can easily be hydrolyzed to glucose and indoxyl. Mild oxidation, such as exposure to air, converts indoxyl to indigo. Indigo treated with sulfuric acid produces a blue-green color. It became available in the mid-1700s. Sulfonated indigo is also referred to as Saxon blue or indigo carmine.

3.3. Isolation of Adapted Bacterial Strains From denim Industry Wastewater

3.3.1. Isolation of indigo degrading organisms by plate assay technique (He Fang et al., 2004)

The spread plate assay method is used for the enumeration of aerobic microorganisms from the given sample. This was done by serial dilution of the samples, placing 0.1ml of the diluted sample in an agar plate containing 0.1 % of indigo carmine and spreading the sample over the surface with the help of an L-rod. The plates were kept for incubation at 37°C as for 24 hours. The cultures which showed a zone of clearance around their colonies were isolated and used for further screening.

3.3.2. Screening of selected bacterial isolates for indigo dye decolorization (Chen et al., 2008)

Due to the insoluble nature of indigo dye in water, indigo carmine was suspended in broth for isolating indigo degrading microorganism (since both indigo and indigo carmine dyes share a common H- chromophore with pie bond). The indigo carmine suspension was prepared by taking 0.1% of the dye in nutrient broth. Five ml of medium was transferred into test tubes. Microbial cells previously isolated were grown overnight in nutrient broth.
The cells from overnight culture (single loopful) were inoculated into the medium. The tubes were kept for incubation at 37°C for 24 hours.

After incubation period, the tubes were checked for the percentage of decolorization for each of the isolated organism. The tubes were vigorously shaken and observed for any biosorption characteristics of the isolated cultures. The cultures showing decolorization were taken and measured by UV-vis spectrophotometer at 609 nm. The decolorization efficiency was expressed in quantitative terms in percentage.

\[
\text{% Decolorization} = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100
\]

3.4. Identification of Selected Microorganisms

The screened bacterial strains were identified by using standard biochemical (Cappucino and Sherman, 1999) and microscopic techniques according to Bergey’s Manual of Systemic Bacteriology (Sneath, 1994).

3.5. Strain Identification (Tamura et al., 2007)

3.5.1. Genomic DNA extraction

The isolated bacterial strain was grown in 2ml Luria Britanni Broth (HiMedia) overnight at 35°C. The culture was spin at 7000 rpm for 3 min. The pellet was resuspended in 400 µl of sucrose TE (Tris EDTA). Lysozyme was added to a final concentration of 8 mg/ml and incubated for 1h at 35°C. To this tube, 100 µl of 0.5M EDTA (Ethylene Diamine Tetra Acetic acid) (pH 8.0), 60 µl of 10% SDS (Sodium Dodesyl Sulphate) and 3 µl of proteinase K from 20 mg/ml were added and incubated at 55°C overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile buffer.

3.5.2. 16S ribosomal RNA (rRNA) gene sequencing

PCR on the extracted DNA was performed in a 100-µl volume. Oligonucleotide primers with specificity for eubacterial 16S rRNA genes, primers 16S rDF (5'-AGAGTTRGTATCMYGCTWAC-3'); 16S rDR (5'-CGYTAMCTTWTTACGRCT-3') were used to amplify the 16S rRNA gene fragments with template DNA originating from
bacteria. The following conditions were used for DNA amplification: 25 cycles consisting of denaturation at 94°C for 1 min, annealing at 50°C for 5 sec and extension at 60 °C for 4 min followed by a final extension at 72°C for 3 min. Amplified PCR products of the proper size (about 1500 base pair fragment) were confirmed by electrophoresis of 10 μL sub samples through a 1% horizontal agarose gel containing 0.5 μg/mL ethidium bromide. Gels were examined under UV light and photographed.

3.5.3. Nucleotide sequence analysis

PCR products were purified using QIAquick Spin columns (Qiagen Inc., Chatsworth, CA). ABI 3130 genetic analyzer in combination with Big Dye Terminator version 3.1 Cycle Sequencing Kit was used for sequencing the purified PCR products by the help of Chromous Biotech centre at Bangalore. Nucleotide sequences were compared with sequences in the National Centre for Biotechnology information (NCBI). GenBank database using the BLASTn program and Ribosomal Database Project (RDP) database using the sequence matching program (http://www.ncbi.nlm.nih.gov/Blast).

3.6. Compatibility Analysis

Prior to the development of a bacterial consortium the selected cultures should be analyzed for its compatible nature. Four nutrient agar plates were taken and each plate was bored with three wells. The first plate was smeared with one of the four selected culture (PK28) and added with 10μl of the supernatant from PK07, PK18 and PK23 in the three wells respectively. The plates were then kept for incubation at 37°C for 24 hours. Absence of any zone of inhibition around the wells showed that the cultures are compatible. The test was repeated by changing the swabbed organisms with the other three selected bacterial isolates and changing the supernatants from the organisms added in the bored wells accordingly (Ammar et al., 1998).

3.7. Characterization of Untreated Denim Industrial Effluent

The raw effluent was characterized by measuring the values of 11 different physico-chemical parameters. These parameters (Total Dissolved Solids (TSS), Total Solids (TS), Total Dissolved Solids (TDS), Chemical Oxygen Demand (COD), pH, color, turbidity, hardness, conductivity, resistivity, and alkalinity) were chosen in accordance with the regulations of Tamil Nadu Pollution Control Board. All the mentioned physico-chemical analyses were done immediately after the effluent sample was collected.
3.8. Characterization of polyurethane foam (PUF)

The polyurethane foam sheets were purchased from nearest departmental stores. The following three properties of PUF were assessed to determine whether they were suitable to be used for immobilization of microorganisms and as an indigo dye adsorbent. 3.4.1 Toxicity Test 500 µl of 3-day old liquid cultures of PK07, PK18, PK23 and PK28 were each spread evenly on agar plate. PUF of each density were cut into slices of about 1 cm × 1 cm. Each slice was placed onto the middle of nutrient agar plates already spread with bacterial liquid culture. Plates without PUF were used as a control. The plates were incubated at 37°C for 2 days. The area around and in contact with the PUF were observed for bacterial growth (Jerabkova et al., 1997).

3.9. Growth of Adapted Bacterial strains in Nutrient broth (NB)

The growth curve of each microorganism in NB was determined. Two ml of a 3-day old liquid was inoculated into 20 ml of NB. The cell number was quantified by spread plate on nutrient agar and expressed as colony forming unit (CFU)/ml. To determine if the microorganisms used were able to grow in a textile effluent containing environment, their growth curve was also obtained. The method was similar to determining growth curve in NB. This experiment was repeated twice.

3.10. Toxicity Test for PUF

About 500 µl of 2 day old liquid cultures of PK07, PK18, PK23 and PK28 were each spread evenly on agar plate. The PUF of each density were cut into slices of about 1 × 1 cm. Each slice was placed onto the middle of nutrient agar plates already spread with bacterial liquid culture. Plates without PUF were used as controls. The plates were incubated at 37°C for 24-48 hours. The area around and in contact with the PUF were observed for bacterial growth.

3.11. Immobilization of Cells

3.11.1 Enumeration of viable cells immobilized on polyurethane foam (PUF)

Polyurethane foam cubes were weighed and placed into a 100 ml of conical flask containing 20 ml of nutrient broth and autoclaved at 121°C for 15 min, two ml of 3 day old cultures were then inoculated separately into each flask. The cultures were incubated at 30°C with orbital shaking (120 rpm) for ten days. Enumeration of viable cells was carried out 2, 4, 6, 8 and 10 after incubation. To ensure that only the immobilized cells were further
quantified, the PUF was first rinsed with sterile nutrient broth. It was torn into pieces using sterile forceps and then suspended in nutrient broth and vortexed to dislodge the immobilized cells. About 0.1 ml aliquots were spread onto nutrient agar plate and incubated at 37°C until colonies appeared. The experiment was repeated four to five times. The attachment efficiency (AE) was calculated as the fraction of the total viable cells that was immobilized (Jerabkova et al., 1997).

3.12. Treatment Trials of Denim Industry Wastewater
Denim industry wastewater containing indigo dye was treated using,

- Pure broth cultures
- Ca-Alginate immobilized adapted bacterial cells
- Cultures immobilized onto inert polyurethane foam cubes (PUF)

3.12.1. Treatment of Denim industrial waste using pure broth cultures
To 95 ml of the effluent sample, 5ml of individual cultures and consortium (composed of all the screened cultures in equal concentration) were inoculated. After inoculation the samples were incubated in a metabolic shaker (120 rpm) for 24 hours at room temperature for a period of 5 days. Samples were retrieved from the flasks after 5 days of incubation and the bioremediation efficiency of the individual cultures as well as that of the consortium were studied by analyzing the physico-chemical parameters (Idaka et al., 1987).

3.12.2. Treatment of Denim industrial waste using Ca-alginate immobilized cells
The Ca-alginate entrapment of bacterial cells was performed as per the method described by Kierstan and Coughlan (1985). About 4% sodium alginate was prepared in distilled water and the alginate solution and cell suspension (bacterial consortium) were mixed in an equal volume and the mixture was extruded drop by drop into a 0.2M CaCl₂ solution through a sterile pipette and the gel beads of 3 mm diameter were obtained. Three gram of beads was measured (in wet condition) and it was added to 97 ml of denim textile effluent. This was incubated at room temperature in a metabolic shaker at 120rpm for 5 days. Sample without inoculation was also used as a control. The treated effluent samples were retrieved at the end of 5th day and all the physico-chemical parameters were analyzed for the bioremediation efficiency.
3.12.3. Treatment of Denim industrial waste using bacterial strains immobilized on inert polyurethane foam

Polyurethane foam was used in the development of biofilm (Fig 14) which offers excellent conditions for microbial growth and retention, due to matrix formation. The quick of biofilm formation is another major advantage since it needs only a low level of microbial organization. Polyurethane foam was sterilized using water at 100°C for 20 minutes and the excess water was drained off after sterilization. The pure adapted bacterial broth culture of individual selected cultures should be added in such a way that the foam gets completely immersed in it. It was incubated for 24 hours at room temperature for the biofilm to develop in the matrix of the polyurethane foam by incubating it in a metabolic shaker (120 rpm). After incubation the excess broth present along with polyurethane foam was drained off and to this 100 ml of denim industry waste effluent sample was added. The samples were incubated in a metabolic shaker (120 rpm) for a period of 5 days at room temperature. Samples were retrieved from the flasks after 5 days of incubation and the bioremediation efficiency of the individual cultures as well as that of the consortium were studied by analyzing the physico-chemical parameters.

![Image of biofilm development on inert PU foam]

**Figure 14: Bacterial biofilm development on inert PU foam**

3.13. Bioremediation Assays

3.13.1. Analysis of physico-chemical parameters

a) Color

Color was measured by an UV-Vis spectrophotometer at a wavelength of 609nm in which maximum absorbance spectra was obtained to the effluent sample. Because of the insoluble nature of indigo in water, it should be treated with concentrated sulfuric acid for 30 minutes in a water bath at 80°C before measuring the OD value (Gutievrez *et al.*, 1990).
b) Turbidity

Turbidity was measured by an UV-Vis spectrophotometer at a wavelength of 609 nm (SL244BB-Elico) (Amar et al., 2010).

c) pH

The pH was measured using a digital calibrated pH meter.

d) Chemical Oxygen Demand

Sample of the effluent was first analyzed for COD immediately after collection. The biologically treated sample was also analyzed for COD as earlier reported (Asamudo et al., 2005). In COD flask, 20 ml of the sample was mixed with about 10ml of 0.025 N potassium dichromate solution and 30ml of concentrated sulphuric acid was added to the side of the flask. To this a pinch of silver sulphate and mercuric sulphate were added to oxidize the nitrogenous compounds and to get rid of the intruding chlorine particles respectively. This was then refluxed in a water bath at 100°C for 2hours in a closed environment. After this 2 to 3 drops of ferroin indicator was added and titrated with 0.1N ferrous ammonium sulphate (FAS) solution. The end point was characterized by the formation of brick red color (APHA, 1992).

\[
COD \text{ mg/l} = \frac{(\text{Blank-sample}) \times \text{Normality of FAS} \times 8000}{\text{Volume of sample taken}} \times \text{Dilution factor}
\]

e) Total Solids (TS)

Total solids (TS) were determined gravimetrically by evaporating a known volume of effluent sample to dryness in a pre-weighed crucible on a muffle furnace at 350°C (APHA, 1989; Ademoroti, 1996). TS was determined by using the following formula,

\[
TS \text{ mg/l} = \frac{\text{Final wt (in gm)} - \text{initial wt (in gm)}}{\text{Volume of sample taken}} \times 1000 \times 1000
\]

f) Total Suspended Solids (TSS)

A total suspended solid consists of silt clay fine particles of organic and inorganic matter. TSS was determined by filtering a known volume of sample through a pre-weighed filter paper. The filter paper was then dried at 103-105°C (APHA, 1989; Ademoroti, 1996). TSS was determined by using the following formula,
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g) Total Dissolved Solids (TDS)

TDS was determined by taking a known volume of sample in a pre-weighed bottom of the petri dish. The petri dish was kept at a 160-180°C for drying of the dissolved solids in a sample (APHA, 1989; Ademoroti, 1996). TDS can be determined by using the following formula,

\[
TSS \text{ mg/l} = \frac{\text{Final wt (in gm)} - \text{initial wt (in gm)}}{\text{Volume of sample taken}} \times 1000 \times 1000
\]

h) Hardness

Hardness of the effluent may be due to the accumulation of salts from contact with soil and geological formations or it may be due to pollution by industrial effluents. Hardness was determined by EDTA titrimetric method in which, 5ml of sample and 2ml of ammonia solutions were mixed and titrated with N/100 of EDTA with a pinch of Erichrome black-T as an indicator. The end point was characterized by the change in color from a wine red to a blue color. Hardness of the sample can be determined by using the following formula (APHA, 1992).

\[
\text{Hardness mg/l} = \frac{\text{Volume of EDTA}}{\text{Volume of sample}} \times 1000
\]

i) Conductivity, Resistivity and Salinity

The textile parameters like Conductivity, Resistivity and Salinity (Alkalinity) were monitored by using calibrated Water proof portable meter (Cyber Scan Series 600).

3.14. Optimization of Cultural Conditions For Efficient Bioremediation process (Khlifi et al., 2010)

At first the efficient adapted bacterial combination which had been immobilized on polyurethane foam was selected under different optimized conditions such as retention time, initial pH, incubation temperature, different substrate (dye) concentration, different foam
concentration, biofilm formation at different incubation time and its efficiency in degradation.

3.14.1. Effect of Retention time on bioremediation of denim industry waste

To about 1.5 g of foam, consortium of screened bacterial cultures was inoculated until the foam gets immersed in it. It was incubated for 24 hours at room temperature for biofilm development in a metabolic shaker (120 rpm). After the given incubation time the excess broth present along with polyurethane foam was drained off and to this 100ml of effluent sample was added and kept for five days of incubation. Sample was retrieved after each day of incubation and the bioremediation ability of the consortium was studied for 5 days by measuring the various physico-chemical parameters (COD, Hardness, TS, TSS, TDS, pH, Color, Salinity, Conductivity, Resistivity and Turbidity). The optimum retention time for the consortium could be found out by measuring the reduction in the parameters during the specified period of incubation.

3.14.2. Effect of Initial pH on Bioremediation of Denim Industry Waste

To about 1.5 g of foam, consortium of screened bacterial cultures was inoculated until the foam gets immersed in it. It was incubated for 24 hours at room temperature for biofilm development in a metabolic shaker (120 rpm). After the given incubation time the excess broth present along with polyurethane foam was drained off and to this 100ml of effluent sample was added. Now the effluent was adjusted at varying pH ranges (5, 6, 7, 8, and 9) and incubated at room temperature for 24 hours under shaking conditions (120rpm). Following incubation the samples were retrieved from day 1 upto day 5 and on each of these days the bioremediation ability of the consortium was studied by measuring the various physico-chemical parameters (COD, Hardness, TS, TSS, TDS, pH, Color, Salinity, Conductivity, Resistivity and Turbidity). The optimum pH for the consortium could be found out by measuring the reduction in the parameters after 5 days of incubation.

3.14.3. Effect of Initial organic loading rate on Bioremediation of Denim Industry Waste

To about 1.5 g of foam, consortium of screened bacterial cultures was inoculated until the foam gets immersed in it. It was incubated for 24 hours at room temperature for biofilm development in a metabolic shaker (120 rpm). After the given incubation time the excess broth present along with polyurethane foam was drained off and to this various
concentrations (volume) of effluent sample was added (20%, 40%, 60%, 80%, and 100%) and incubated in room temperature for 24 hours under shaking conditions (120rpm). Following incubation the samples were retrieved from day 1 upto day 5 and on each of these days the bioremediation ability of the consortium was studied by measuring the various physico-chemical parameters (COD, Hardness, TS, TSS, TDS, pH, Color, Salinity, Conductivity, Resistivity and Turbidity). The optimum initial substrate concentration for the consortium could be found out by measuring the reduction in the parameters after 5 days of incubation.

3.14.4. Effect of Incubation Temperature on Bioremediation of Denim Industry Waste

To about 1.5 g of foam, consortium of screened bacterial cultures was inoculated until the foam gets immersed in it. It was incubated for 24 hours at room temperature for biofilm development in a metabolic shaker (120 rpm). After the given incubation time the excess broth present along with polyurethane foam was drained off and to this 100ml of effluent sample was added and kept at varying temperature (7°C, 17°C, 27°C, 37°C, and 47°C) and incubated at room temperature for 24 hours under shaking conditions (120 rpm). Following incubation the samples were retrieved from day 1 upto day 5 and on each of these days the bioremediation ability of the consortium was studied by measuring the various physico-chemical parameters (COD, Hardness, TS, TSS, TDS, pH, Color, Salinity, Conductivity, Resistivity and Turbidity). The optimum incubation temperature for the consortium could be found out by measuring the reduction in the parameters after 5 days of incubation.

3.14.5. Effect of the volume of inert substrate (PU foam) on Bioremediation of Denim Industry Waste

To various amounts of foam (0.5 g, 1 g, 1.5 g, 2 g, 2.5 g [Wt/V]) consortium of screened bacterial cultures was inoculated until the foam gets immersed in it. It was incubated for 24 hours at room temperature for biofilm development in a metabolic shaker (120 rpm). After the given incubation time the excess broth present along with polyurethane foam was drained off and to this 100ml of effluent sample was added and incubated in room temperature for 24 hours under shaking conditions (120 rpm). Following incubation the samples were retrieved from day 1 upto day 5 and on each of these days the bioremediation ability of the consortium was studied by measuring the various physico-chemical parameters.
(COD, Hardness, TS, TSS, TDS, pH, Color, Salinity, Conductivity, Resistivity and Turbidity). The optimum foam concentration for the consortium could be found out by measuring the reduction in the parameters after 5 days of incubation.

3.14.6. Effect of incubation time for biofilm development on bioremediation of denim industry waste

To about 1.5 g of foam, consortium of screened bacterial cultures was inoculated until the foam gets immersed in it. The biofilm was allowed to develop over increased periods of incubation time (1\textsuperscript{st} day, 2\textsuperscript{nd} day upto 5\textsuperscript{th} day) under shaking conditions in a metabolic shaker (120 rpm). After the given incubation time the excess broth present along with polyurethane foam was drained off and to this 100ml of effluent sample was added and incubated in room temperature for 24 hours under shaking conditions (120 rpm). Following incubation the samples were retrieved from day 1 upto day 5 and on each of these days the bioremediation ability of the consortium was studied by measuring the various physico-chemical parameters (COD, Hardness, TS, TSS, TDS, pH, Color, Salinity, Conductivity, Resistivity and Turbidity). The optimum reduction was seen in biofilm developed in any specified day of incubation.

3.15. Treatment of Textile Effluent under Optimized Cultural Conditions

About 1.5 g of the foam was taken and allowed to develop biofilm for 1 day. A substrate concentration of 100\% was added and incubated at 27\°C for 7 days. The pH of the effluent was adjusted to pH 6. The sample was also checked quantitatively for the presence of toxic intermediates with the help of GC-MS.

3.16. Characterization of Indigo Dye Degrading Compounds Through GCMS

GC-MS is particularly useful for identification of products from disperse vat dyes. Treated and untreated effluents were centrifuged. Equal volumes of the supernatant collected were mixed with diethyl ether separately in order to retrieve the organic content of the treated and untreated effluent samples. The organic layer was then collected and allowed to evaporate at room temperature. The residue that remains was then suspended in 5ml 100\% methanol. The methanol solution was then used for GC-MS analysis for both the treated and untreated effluent samples separately. GC-MS was performed using a THERMO GC - TRACE ULTRA VER: 5.0, THERMO MS DSQ II. Identification of degradation products were made by comparison of retention time and fragmentation pattern with known products.
reference compounds as well as with mass spectra stored in the library search results in the computer software (version 1.10 beta, Shimadzu) of the GC-MS (Adosinda et al., 2003).

3.17. Scanning Electron Microscope (SEM) Analysis

The samples of PUF from the bioreactor before and after the effluent treatment were processed. Biofilm developed polyurethane foam was taken and air dried and SEM analysis was done. The foam materials used were having irregular and porous surface providing large surface area for immobilizing bacterial cells for biofilm formation. The bacterial biofilm consisted of heterogenous population of short and long rods, cocci and filamentous. This was also done to study the colonization of the bacterial biofilm and to detect whether the effluent has any toxic effect over the bacterial cultures and to check any morphological changes. The scanning electron micrographs were taken on Jeol JSAL 6360.

3.18. Cytotoxicity Analysis

HBL 100 cells were purchased from National Centre for Cell Science (NCCS), Pune and maintained in DMEM and McCoy's medium, supplemented with non essential amino acids and this experiment was done at King Institute of Preventive Medicine (Chennai). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2 in a CO2 incubator. About 1×10^4 cells/wells were seeded into 96 well tissue culture plates and incubated for 48h. HBL 100 cells were treated with 50 µL concentration of degraded indigo dye and indigo dye. As a control for bacterial toxins, solutions that had been incubated with bacteria but without indigo dye and then filter sterilized were also added to the cells at an equivalent volume. After exposure to the cells for varying time periods up to 48 h, the medium containing the dead, floating cells was removed and the adherent cells were detached from the plate. The treated cells were incubated for 48h for cytotoxicity analysis. The cells were then subjected for MTT assay.

The stock concentration (5mg/mL) of MTT-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) was prepared and 200µL of MTT was added in each degraded indigo dye treated wells and incubated for 4h. Purple color formazone crystals were observed and these crystals were dissolved with 100µL of dimethyl sulphoxide (DMSO), and read at 620 nm in a multi well ELISA plate reader (Thermo, Multiskan). OD value was subjected to sort out percentage of viability by using the following formula,
3.19. Comparison of Adsorption isotherm kinetics on Bioremediation of denim industry waste by free bacterial cells (consortium), Ca-alginate immobilized cells and PU foam fixed bacterial consortium

Adsorption isotherms were compared with adapted bacterial broth cultures, Ca-alginate immobilized cells as well as the bacterial consortium fixed inert PUF to evaluate the dye adsorption capacity of denim industrial wastewater. This test was required to evaluate Langmuir and Freundlich adsorption isotherm kinetics to determine the adsorption equilibrium in denim wastewater solution for the approximation of obtained results. The constant in the *Langmuir isotherm* was determined by plotting \( \frac{C_e}{x/m} \) versus \( C_e \) and making use of Eq. 1 written as,

\[
\frac{C_e}{x/m} = \frac{1}{a} + \frac{1}{a C_e} 
\]

\[ (1) \]

\( x/m = \text{mass of g adsorbate (PUF)} \)

\( a,b = \text{empirical constant} \)

\( C_e = \text{equilibrium concentration of adsorbate in solution after adsorption (mg/L)} \)

In *Freundlich isotherm* was determined by plotting \( \frac{C_e}{x/m} \) versus \( C_e \) and making use of Eq. 2 written as,

\[
\log (x/m) = \log K_f + \frac{1}{n} \log C_e 
\]

\[ (2) \]

To study the adsorption kinetics of the free bacterial cells and the bacterial consortium fixed on polyurethane foam, two kinetic models were used that include Lagergren and pseudo-second order models. The linearized form of Lagergren (Eq. 3) (Lagergren and Svenka, 1898) and pseudo-second order (Eq. 4) (Ho and McKay, 1998a) models can be expressed as:

i) **Lagergren (Pseudo first order)**

\[
\log (q_e - q) = \log q_e - \frac{K_{ad}}{2.303 \times t} 
\]

\[ (3) \]

Whereas, \( q \) and \( q_e \) are amounts of adsorbate adsorbed (ml/L) at time, \( t \) (hrs) and at equilibrium respectively. \( K_{ad} \), is the adsorption rate constant (1/hrs).

ii) **Pseudo-second order**

\[
\frac{t}{Q_t} = \frac{1}{K_1 Q_e^2} + \frac{1}{Q_e} \times t 
\]

\[ (4) \]
Where, \( Q_e \), is the amount of adsorbate adsorbed at equilibrium (ml/L)

\( Q_t \), is the amount of adsorbate adsorbed at time \( t \) (hrs)

\( K_1 \), is the second order equilibrium rate constant (ml/hrs).

3.20. Mathematical Modelling on Bioremediation Efficiency of Multiple Species Used as Biobarrier Model

The microbes do not use indigo dye as a source but the amount of indigo dye degraded depends on the amount of enzymes produced, which depends directly on the amount of carbon and nitrogen supplements consumed and also calculated the rate of indigo dye degradation per substrate consumption under aerobic condition. We model the water flow, the transport of nutrients and degradation of indigo dye as well as the growth of biofilm forming bacteria and biodegradation strains. The limiting nutrients in the simulations and assume that all the other nutrient present in enough quantities. In this section we show qualitative results of some dual species biobarrier simulations. The purpose and multispecies biofilm experiments that could lead to the design of more effective bioremediation techniques.

In order to model multi-species biofilm interactions in porous media we consider a three-phase mixture consisting of a liquid phase, a solid rock phase and a biofilm phase. Even though the biofilm can be considered to be part of the solid phase, it is simpler to take it as a separate phase. The four molecular species present in the porous medium are the contaminant, the contaminant-reducing microbe unable to form a significant biofilm, the strong biofilm-forming bacteria, the nutrient and the water and rock species. The formulations used in the rest of the paper are valid for diverse species of microbes and chemical substances. The biofilm forming microbes use the nutrients to grow and to form extra polymer substances (EPS). The EPS is used to link the microbes together to form the biofilm. One modeling possibility is to consider the EPS an additional species, but this method increases the number of equations and the complexity of the problem. Since the biofilm microbes use a percentage of the available nutrients to form EPS, a second alternative is to consider the microbes and the EPS as one species. The growth parameters for these microbes have to be adjusted accordingly. The second way and consider that the mass of the biofilm forming microbes includes the EPS. The fundamental equation for saturated transient ground-water flow of constant density, in horizontal direction (1),
3.21. Treatment trial on Crude Indigo dye Decolorization by Adapted Bacterial Supernatant

3.21.1. Comparative studies on Indigo dye decolorization by mixed and individual bacterial supernatant

An assay mixture of indigo dye (100 mg/ml) containing 0.1M phosphate buffer (pH 6) and 4 ml of bacterial supernatants (each individual broth culture) as well as the mixed bacterial supernatants (the culture supernatant from each selected strain was mixed in equal volume to make a total quantity of 4 ml) were also incubated in metabolic shaker (120 rpm) at 27°C. The degree of decolorization of the dye was analyzed at different time intervals (in hours) like 8, 16, 24, 32, 40, 48, 56, 64 and 72 respectively. These aliquots were checked the decolorization pattern of the mixture of bacterial supernatant with respective time intervals. After incubation the samples containing the assay mixture was analyzed by UV-vis spectrophotometer at 609 nm (SL244BB-Elico). These treated samples were then lyophilized in Mini Lyodel Freez dryer lyophilizer.

3.21.2. Optimization of cultural conditions for Indigo dye decolorization efficiency

A loop full of selected bacterial strains were grown in nutrient broth and incubated at 27°C for 24-48 hrs in a metabolic shaker (120 rpm). The crude bacterial supernatant was collected after removal of cell debris from the broth solution by centrifuging at 10,000 rpm and 4°C for 15 minutes. In this study, the decolorization experiments were run under various cultural conditions using traditional stepwise protocol i.e., varying a factor at a time and keeping others constant for the optimization of dyes concentration (50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L and 250 mg/L), pH (4, 5, 6, 7, 8 and 9) (adjusted with 1N HCl or 1N NaOH), Temperature (17°C, 27°C, 37°C, 47°C, 57°C, 67°C and 77°C) and different additional Carbon (glucose, fructose, lactose and maltose) sources at 0.1 g/L and Nitrogen sources (ammonium chloride, urea, peptone and yeast) at 0.1 g/L were added to study their effect on dye decolorization rate under previously optimized conditions. Culture flasks were kept for complete agitation in shaker for 120 rpm for 48 h.
3.21.2.a Effect of initial pH on indigo dye decolorization

The adapted bacterial strains were grown in 25 ml one quarter strength nutrient broth medium in a 250 ml baffled Erlenmeyer flask at 30°C for 24 hours with rotation at 120 rpm. These variables were selected on the basis of convenience. Cells were pelleted by centrifugation (10,000 x g for 30 minutes) at 4°C and the supernatant was collected and filtered through 0.2 µm pore size filter. Take 1 ml of culture supernatant form individual bacterial strain was taken and inoculated into the indigo dye containing (100 mg/L) 0.1M potassium phosphate buffer which has further been incubated at pH 4, 5, 6, 7, 8 and 9. Aliquots of the culture media were withdrawn at 48h and the decolorization was determined by measuring the absorbance of decolorization medium at 609 nm.

3.21.2.b Effect of Temperature on indigo dye decolorization

The adapted bacterial strains were grown in 25 ml one quarter strength nutrient broth medium in a 250 ml baffled Erlenmeyer flask at 37°C for 24 hours with rotation at 120 rpm. Cells were pelleted by centrifugation (10,000 x g for 30 minutes) at 4°C and the supernatant was collected and filtered through 0.2 µm pore size filter. About 1 ml of culture supernatant form individual bacterial strain was inoculated into the indigo dye (100 mg/L) containing 0.1 M potassium phosphate buffer which has further been incubated at different temperatures like 7°C, 17°C, 27°C, 37°C and 47°C for 48 hours. Aliquots of the culture media were withdrawn at 48h and the decolorization was determined by measuring the absorbance of decolorization medium at 609 nm.

3.21.2.c Effect of Carbon substrates on indigo dye decolorization

The adapted bacterial strains were grown in 25 ml one quarter strength nutrient broth medium in a 250 ml baffled Erlenmeyer flask at 37°C for 24 hours with rotation at 120 rpm. Cells were pelleted by centrifugation (10,000 x g for 30 minutes) at 4°C and the supernatant was collected and filtered through 0.2 µm pore size filter. About 1 ml of culture supernatant form individual bacterial strain was inoculated into the indigo dye (100 mg/L) containing 0.1 M potassium phosphate buffer (basal medium). Various carbon sources viz. glucose, fructose, sucrose and lactose at 0.1% (w/v) were individually added in the basal medium and inoculated with 4% (v/v) of bacterial cultures separately with their respective optimized pH (6), temperature (47°C) then incubated for 48h for decolorization.
Aliquots of the culture media were withdrawn at 48h and the decolorization was determined by measuring the absorbance of decolorization medium at 609 nm.

### 3.21.2.d Effect of Nitrogen substrates on indigo dye decolorization

The adapted bacterial strains were grown in 25 ml one quarter strength nutrient broth medium in a 250 ml baffled Erlenmeyer flask at 37°C for 24 hours with rotation at 120 rpm. Cells were pelleted by centrifugation (10,000 x g for 30 minutes) at 4°C and the supernatant was collected and filtered through 0.2 µm pore size filter. About 1 ml of culture supernatant form individual bacterial strain was inoculated into the indigo dye (0.1%) containing 0.1 M potassium phosphate buffer. Various Nitrogen sources viz. Urea, Ammonium chloride, Peptone and Yeast at 0.1% (w/v) were individually added in the basal medium and inoculated with 4% (v/v) of bacterial cultures separately with their respective optimized pH (6), temperature (47°C) then incubated for 48h for decolorization. Aliquots of the culture media were withdrawn at 48h and the decolorization was determined by measuring the absorbance of decolorization medium at 609 nm.

### 3.21.2.e Effect of Initial indigo dye concentration

The adapted bacterial strains were grown in 25 ml one quarter strength nutrient broth medium in a 250 ml baffled Erlenmeyer flask at 37°C for 24 hours with rotation at 120 rpm. Cells were pelleted by centrifugation (10,000 x g for 30 minutes) at 4°C and the supernatant was collected and filtered through 0.2 µm pore size filter. When the effect of different concentrations of Indigo dye (50, 100, 150, 200 and 250 mg/L) on decolorization was observed by taking the 4 ml of mixed bacterial supernatant then inoculated into each 100 ml of 0.1M phosphate buffer containing conical flasks at different concentrations of Indigo dye. Aliquots of the culture media were withdrawn at 48h and the decolorization was determined by measuring the absorbance of decolorization medium at 609 nm.

### 3.21.3. Decolorization of indigo dye under optimized cultural conditions

The basal medium of Indigo dye (100 mg/L) decolorization with respective pH (6), temperature (47°C), suitable Carbon (glucose-0.1%) and Nitrogen (urea-0.1%) supplements and were incubated at 48 h under all the above cultural conditions. The decolorization efficiency was monitored by measuring the absorbance of decolorization medium at 609 nm (UV-vis spectrophotometer).
3.22. Purification of Indigo dye Degrading Gene of Interest (ThrH gene product) From *P. aeruginosa* PA01.

3.22.1. Ammonium sulphate precipitation

The adapted bacterial strains were grown in sterile nutrient broth medium for 24 hours. Cells were harvested and centrifuged (10,000 x g for 30 minutes at 4°C). The supernatant was then filtered using 0.45 µm pore size filters. Ammonium sulfate was poured slowly into the supernatant over a period of ten minutes, allowing the salt to slowly dissolve. The supernatant was continually stirred at room temperature for an additional 25 minutes. Precipitates were recovered by centrifugation (10,000 x g for 10 minutes at 25°C), and dissolved in 10mM Tris-HCl, 10mM NaCl buffer (pH 8.0). Each (NH₄)₂SO₄ fraction was dialyzed overnight against the same buffer (1:50 volume). The protein concentration was measured and specific activity calculated.

3.22.2. Dialysis: Preparation of dialysis tubing

The tubing was cut into pieces of required and convenient length and allowed to boil for 10 minutes in a range volume of sodium bicarbonate buffer. The tubing was rinsed thoroughly in distilled water. Again it was allowed to boil in 1mM EDTA (pH 8). The tubing was cooled down and stored at 4°C in 50% ethanol. Care was taken that the tubing was thoroughly submerged. After this step, the tubing was handled with gloves. Before use, the tubing was washed inside and outside with distilled water.

3.22.3. Desalting of bacterial enzyme fractions

One end of the dialysis tube was tied with a thread and ensured it was leak proof. The pooled protein (amino acid) fraction obtained from bacterial broth culture was transferred to an active dialysis bag. The contents were pooled into the dialysis bag and tied after including some air and twisting the open end of the dialysis bag. The bags containing pooled contents were dispensed into 1-2 ml of phosphate buffer. The setup was continuously stirred by means of a magnetic stirrer and the temperature was maintained at 4°C. The dialysis was carried out over 16 hours with over two changes of buffer. About 2-4 drops of Nessler’s reagent was added to check the completion of dialysis which is indicated by the absence in formation of brown precipitate. This is to test the ammonium in the aqueous solution (buffer). The contents were dispensed into tubes after dialysis. The protein was
taken for further purification using DEAE Sepharose A-50 ion exchange column chromatography.

3.22.4. Determination of protein

The total protein content was estimated by the method described by Lowry et al., (1956). A quantity of 10mg bovaine serum albumin (BSA) was dissolved in 10ml of distilled water and used as stock solution. To a series of clean test tube, 0.2 - 1.0 ml of BSA (stock solution) was added and made up to final concentration of 5 ml with distilled water. From these dilutions 0.2ml was taken in to different test tubes and 2.5ml of Alkaline Copper Sulphate solution was added to each test tube and incubated for 10 minutes at room temperature. After incubation, 0.5ml of Folin-Ciocalteau reagent was added to each tube and incubated under dark condition for 30 minutes at room temperature. The OD was read at 260 nm against the reagent blank and was plotted on a graph. Thus the standard graph was obtained.

3.22.5. DEAE Sepharose A-50 ion exchange column chromatography

The bacterial enzyme was purified by DEAE sephadex ion exchange column chromatography. About 25-30g of DEAE sephadex A-50 cellulose was added to a beaker containing equal volume of 0.1N NaOH, stirred well in such a way that no air bubbles were formed and left at room temperature for 30 minutes. The supernatant was decanted and the sediment was treated with distilled water, stirred well and left at room temperature for 30 minutes. This was repeated several times until there was a neutral reaction. Further, the sediment was mixed with equal volume of 0.1N HCl was left at room temperature with intermittent stirring for 30 minutes. This was further washed several times with distilled water until there was a neutral reaction.

3.22.6. Packing of chromatograph column

For purification of protein, the column size 2cm was used. The burette was first cleaned well and it was packed first with glass wool to form an even bed and a rubber tube with pinch cork was attached to tip of the burette. The column was fixed to stand in vertical position. The DEAE slurry was poured into the column along the side to avoid air bubbles and was allowed to settle. Once the column was set, it was equilibrated with 25mM phosphate buffer at pH 8.
3.22.7. Elution of bacterial protein fraction

Once the column was equilibrated (25 mM phosphate buffer, pH 8) the level of buffer in column was allowed to run down to the matrix and the outlet was closed. The bacterial protein sample was layered on the top of the column and was allowed to run till all the samples had entered the glass bed. Then a continuous constant flow of 25 mM phosphate buffer was maintained until all the protein came out. Bacterial protein was eluted with 250 mM phosphate buffer at pH 8.

3.22.8. Studies on Indigo dye decolorization Using specific adapted bacterial enzymes

About 100 mg/L of indigo dye was mixed with 0.1 M phosphate buffer at pH (6) and added 0.5 ml of bacterial pooled fractions then the mixture was incubated at 47°C for 24 hours. The liquid above the solution was removed and added equal volume of sulfuric acid and water (1:1) and kept under water bath for 10 minutes for the dissolving of indigo dye particles as well as the deactivation of enzyme. The absorbance of combined fractions of extracts was measured at 609 nm and then the decolorization pattern of indigo dye was calculated.

The pooled bacterial fractions from other proteins were separated by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% of stacking and 10% of separating gel. The samples were taken (20 µl) and treated with an equal amount of sample treated buffer consisting of 5% mercaptoethanol, 2% SDS, 0.005% bromophenol blue dye in 0.05 M Tris buffer pH 8.

The samples were loaded into samples wells along with a known molecular weight markers (low and mid range markers, Genei Pvt, Ltd, Bangalore) and the electrophoresis was performed at 100V current in a Tris glycine system. When bromophenol dye reached at the bottom of the gel, the electrophoresis was stopped. The gel was removed and was stained with coomassie brilliant blue R-250 for 30 minutes. The gel was rinsed with distilled water and destained. The molecular weight of the proteins resolved estimated in comparison to the molecular weight markers.
**Fractionation flow chart**

**Grow 25 ml of each individual bacterial strains**

1. **Take 25 ml of individual culture**  
   [Centrifuged 25 ml (5,000 rpm for 20 minutes at 4°C)]

   - **Cells**
   - **Supernatant** → **Filtered Supernatant (0.2 µm)**

2. **Suspended in 100 ml of PO₄ buffer**  
   Centrifuged 100 ml ((5,000 rpm for 20 minutes at 4°C)]

3. **Washed cells with PO₄ buffer**

4. **[Centrifuged 25 ml (5,000 rpm for 20 minutes at 4°C)]**

5. **Crude extract + Pellet I**

6. **[Centrifuged 25 ml (5,000 rpm for 20 minutes at 4°C)]**

7. **Crude extract + Pellet II**

   - **“Clarification step”**

8. **[Centrifuged 25 ml (5,000 rpm for 20 minutes at 4°C)]**

9. **Pellet in 25 ml of PO₄ Buffer**
3.23. Peptide Sequencing by MALDI-TOF

The resulting trypsinized peptides were dissolved using a 0.5% TFA solution. To reduce chemical background noise for MALDI-MS, sample peptides were purified using Zip-tip C18 (Millipore, Bedford, MA, USA). Peptides were eluted onto the MALDI target plate using a matrix solution containing 10 mg of α-cyano-4-hydroxycinnamic acid dissolved in 1 ml of a solution containing 50% acetonitrile and 0.5% TFA. Sample peptides were analyzed by TOF-MS and TOF-MS/MS mode (4700 Proteomics analyzer, Applied Biosystems, Foster City; CA); UV light (355 nm) of an Nd: YAG laser with a 200 Hz laser repetition rate. For peptide sequencing by MS/MS analysis, collision-induced dissociation was performed using air as the collision gas. The collision energy was set to 1 kV. MS/MS spectra were analyzed using the Mascot program compared with the NCBI nucleotide database.

3.24. N-Terminal Sequence Analysis

The protein spots separated on the gel plate were transferred onto a membrane using a semi dry blotting apparatus (BIO-RAD Trans-Blot SD) at 2 mA/cm² for 45 min and that membrane was stained with Coomassie Brilliant Blue and washed with 50% methanol. Coomassie stained protein spots were excised from the membrane and installed in the blot cartridge of an odel 491A protein sequencer (Perkin–Elmer, Foster City, CA, USA) for sequencing analysis. The obtained N-terminal sequence was used for protein identification by BLAST search of NCBI. This was done at Hyderabad Vimta Lab’s Pvt Lit.

3.25. Methodology for Molecular Docking Study

Molecular modeling studies have been carried out using GLIDE (Grid-based Ligand Docking with Energetics) (Friesner et al., 2004) software version 9.1 developed by Schrodinger running on Red Hat Enterprise Linux 5 (RHEL5) workstation was used for all the steps involved in ligand preparation, protein preparation and Induced Fit Docking (IFD).

3.25.1. Receptor X-ray Structure

The 3D coordinates of the crystal structure of “ThrH” gene product of Pseudomonas aeruginosa was selected as the receptor model in Glide flexible Docking program in Schrodinger. Before Docking all heteroatom’s & water molecules are removed from Protein file 1RKU by using protein preparation wizard application of Schrodinger and missing side chains were filled using prime options of protein preparation wizard (PDB sum). This was
followed by preprocess, analyze, optimization and minimization. These steps were performed to prepare the protein for docking.

3.25.2. Active Site Analysis

The Active sites and their residues obtained from PDB ligand explorer entry of “ThrH” gene product of *Pseudomonas aeruginosa* protein (PDB id-1RKU) (Schrodinger). The entry contains five active site residues (Asp-78, Arg-81, Arg-102, Gly-105, Phe-106).

3.25.3. Ligand Preparation

All the ligands in mol format from Pub Chem compound database are loaded individually in Schrodinger workspace and their geometries are cleaned by clean up geometry option in edit menu. These cleaned ligands in mol format are loaded in the Schrodinger workspace for ligand preparation. In this process Ligands Counter ions and excess hydrogen’s are removed using “desalt” option in task menu of Ligprep application in the Schrodinger (PDB sum). In addition, in presence of OPLS-2005 force field ligfilter is used to produce the optimized ligand structure and also ionizer and generate tautomers options were used to generate possible states of every ligand. The output of this ligprep is in maestro format of every ligand and is used for glide docking with receptor molecule.

3.25.4. Virtual Screening

Docking of the entire (ligand name) compounds against “ThrH” gene product of *Pseudomonas aeruginosa* Protein structure was by using Glide molecular docking program in Schrodinger. The receptor grid can be set up and generated by the Receptor Grid Generation panel of Schrodinger. The shape and properties of the receptor are represented on a grid by several different sets of fields that provide progressively more accurate scoring of the ligands poses. Centroid of selected residues option is used to generate a grid. This option centers grids at the centroid of a set of active site residues. The grid for docking calculations was centered on the five active site residues and the size of the x, y, and z coordinates in receptor grid likely becomes 6.75, 30.87,12.0 Å respectively. Grid generated receptor file is used for ligand docking.

3.25.5. Ligand docking steps was as follows

In Schrodinger glide, submenus of the ligand docking applications are used for docking. High-throughput virtual screening (HTVS) docking is intended for the rapid screening of very large numbers of ligands, Grid generated receptor molecule docked with
ligands is used for HTVS (High-Throughput Virtual Screening). From this screening best ten lowest docked energy ligands were selected for SP docking. Standard-precision (SP) docking is appropriate for screening ligands of unknown quality in large numbers. Ten ligands are selected from HTVS were individually docked to the receptor. Extra-precision (XP) docking and scoring is a more powerful and discriminating procedure, which takes longer to run than SP. XP is designed to be used on ligand poses that have a high score using SP docking. First the database compounds are run through SP docking, then the top 10% to 30% of final poses are taken and docked them using XP, so that it gives the best drug candidates that interacted with the receptor to inhibit the process of the target.

3.26. Decolorization assay: UV-visible spectrum analysis

The dye decolorization was detected by UV-Vis spectrophotometer at Indigo dye λmax (609 nm) using the supernatant from the liquid culture medium after centrifugation at 10,000 rpm for 10 min. the removal of color was reported as % decolorization (% = A₀ - Aᵣ/A₀× 100, where A₀ and Aᵣ are absorbance of the dye solution initially and at cultivation time (t) respectively). Abiotic control (without bacterial consortium) was always included.

3.27. Analysis of the functional groups by Fourier transforms infrared (FTIR) spectroscopy (Campos et al., 2001).

The infrared spectrum originates from the vibrational motion of the molecule. The vibrational frequencies are a kind of fingerprint of the compounds. This property is used for characterization of organic, inorganic and biological compounds. The band intensities are proportional to the concentration of the compound and hence quantitative estimations are possible.

Description

The interference pattern obtained from a two beam interferometer as the path difference between the two beams is altered, when Fourier transformed, gives rise to the spectrum. The transformation of the interferogram into spectrum is carried out mathematically with a dedicated on-line computer.

The Shimadzu FTIR Spectrum instrument consists of globar and mercury vapour lamp as sources, an interferometer chamber comprising of KBr and Mylar beam splitters followed by a sample chamber and detector. Entire region of 450-4000 cm⁻¹ is covered by this instrument. The spectrometer works under purged conditions. Solid samples are
dispersed in KBr or polyethylene pellets depending on the region of interest. This instrument has a typical resolution of 1.0 cm\(^{-1}\). Signal averaging, signal enhancement, base line correction and other spectral manipulations are possible.

**Instrument details**

- Instrument made by Shimadzu
- Model- IR Affinity I
- Detector- DLATGS

**Sample required**

1:200 (1 mg extract in 200 mg KBr)

**Applications**

Infrared spectrum is useful in identifying the functional group like -OH, CN, -CO, -CH, -NH\(_2\) etc.

### 3.28. Identification of the Structure of the Active Constituents by Nuclear Magnetic Resonance (NMR) spectroscopy

NMR spectroscopy is used to determine the molecular structure based on the chemical environment of the magnetic nuclei like \(^1\)H, \(^{13}\)C, 2D NMR, etc., even at low concentrations. This is one of the most powerful non destructive techniques in elucidating the molecular structure of biological and chemical compounds.

**a) Description**

In NMR spectroscopy, a strong RF pulse excites the entire range of precessional frequencies of a given nuclear species whose time response is known as free induction decay (FID) containing all the information. A Fourier transform of FID gives the NMR spectrum. This technique is used in JEOL GSX 400 NB FT-NMR spectrometer. The spectra of samples containing low abundant nuclei like \(^1\)H, \(^{13}\)C, 2D NMR, etc., are thus easily obtained. Also dynamic studies are possible by relaxation measurements. Homo and hetero \(^1\)H decoupling are also possible.

JEOL GSX 400 NMR operates at 300 MHz (for proton) with a magnetic field of 9.3 Tesla. Hence a supercon magnet is used. A PDP-11/73 computer is an integral part of the instrument for the purpose of Fourier transformation and spectral manipulation. The spectrum is plotted on a HP plotter and data can be obtained on a printer. The probes
available are $^1$H/$^{13}$C combined and multinuclei probe to study the nuclei like $^{23}$Na, $^{27}$Al, $^{43}$Ca, $^{37}$Cl, $^{79}$Br etc.

b) Instrument details

JEOL GSX 400 NB, 300 MHz FT NMR Spectrometer.

c) Sample required

5 mg for $^1$H and 15 mg for $^{13}$C. Solubility: 10 mg/ml for $^1$H and 50 mg/ml for $^{13}$C

Solvents available: CDCl$_3$, D$_2$O, C$_6$D$_6$, CD$_3$COCD$_3$ and DMSO$_6$.

d) Applications

Widely used in organic chemistry, biology, medicine, pharmaceuticals, etc. for characterization of compounds.

3.29. Thin layer chromatography (TLC)

Thin layer chromatography is an easy and highly useful technique in research laboratories to separate and identify unknown compounds. Thin layer chromatography is for the separation of a mixture into individual components using a stationary and mobile phase (Gogna et al., 1992).

a) Principle

The principle of separation is adsorption. One or more compounds are spotted on a thin layer of adsorbent coated on a chromatographic plate. The mobile phase solvent flows through because of capillary action towards the adsorbent. The component with more affinity towards the stationary phase travels slower. The component with lesser affinity towards the stationary phase travels faster. Thus the components are separated on a thin layer chromatographic plate based on the affinity of the component towards the stationary phase.

b) Procedure

The optimized conditions were used for the identification of secondary metabolites present in the treated indigo dye solution. The fractions collected from chromatographic columns were monitored by TLC in different solvent systems as in Table 5. These plates were placed in the solvent chamber containing mobile phase. The solvent was allowed to rise to the maximum height of the TLC plate, then they were removed from solvent chamber, dried and the spots were detected by placing the TLC plates in a chamber containing iodine vapour.
Table 5: Thin layer chromatographic solvent systems for separation of active constituents

<table>
<thead>
<tr>
<th>S. No.</th>
<th>TLC mobile phase (94:4:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Benzene</td>
</tr>
<tr>
<td>2.</td>
<td>Ethanol</td>
</tr>
<tr>
<td>3.</td>
<td>Chloroform</td>
</tr>
</tbody>
</table>

3.30. Chromatographic Analysis of Secondary Products Using LC-MS

About 50 ml of culture sample taken at 48 h centrifuged and filtered through whatman filter paper. The filtrate was then extracted thrice with dichloromethane and evaporated in a vacuum evaporator with 40-50°C water bath, after which the residue was dissolved in acetonitrile and used for LC-MS analysis (Agilent Triple Quadra pole, Software: Mass Hunter® Qualitative Work station, Infusion pump: HARWARD PAKD infusion pump). The flow rate was 0.8 ml/min. the ion trap detector source was used for quantification in negative ionization mode. Operating conditions were dry temperature 325°C, capillary voltage 3500 V, nebulizer 14 psi, dry gas helium 5.0 l/min. Instrument control and data acquisitions were performed with 1100 series data analysis for Mass spectrum (MS).

3.31. Treatment Trials at Industrial Scale

The industrial treatment trial was carried out at KG fabrics (Denim Industry) situated at Perundurai, Coimbatore district for a period of one week and the composite sample was used for further characterization. The denim industrial wastewater was collected from dye bath and it was treated in a 500 liter tank with polyurethane foam (PUF) immobilized cells. This section addresses major considerations for design and selection of treatment processes for Common Effluent Treatment Plants (CETPs) using biofilter (bacterial biofilm development on inert PU foam matrix) wastewater treatment technologies and to treat hazardous industrial wastewater and also should focus on simple technology that is cost-effective, requires low maintenance and has minimal operator requirements. A biofilm reactor consists of a series of corrugated polyurethane disc (1 X 1 Meter) mounted on a horizontal shaft (see figure 15) with 4 liters of inoculum (mixed bacterial consortium).
Figure 15: A schematic diagram of industrial scale up process

The PU foam disc, with approximately 40 percent of the disc immersed in the wastewater and the remaining area exposed to the atmosphere, provide a surface for a bacterial slime layer. The alternating immersion and aeration of a given portion of the disc enhance growth of the attached microorganisms and facilitate oxidation of the dissolved organic and nitrogenous materials in the wastewater, and provide a high degree of waste treatment in a relatively short time. The biofilm reactor was operated for 6 months and the samples were retrieved from every alternative fortnight. Experiments were started in batch mode to immobilize bacterial biomass onto the disks. After immobilization, the growth medium was removed and the decolorization medium was continuously fed into the reactor (hydraulic retention time of 15 days).

The effect of Retention time (15\textsuperscript{th} day), pH (6), Temperature (27°C), Initial organic loading rate (100\%), Volume of inert PU foam substrate (1.5g), Incubation time for biofilm development (one day) of bacterial consortium has already been optimized. The bioremediation and biodecolorization efficiency were analyzed by various physico-chemical parameters like COD, Hardness, TS, TSS, TDS, pH, Color, Salinity, Conductivity, Resistivity and Turbidity.
3.32. Statistical Analysis

To evaluate the influence of nature, the various physico-chemical parameters and the influence of the supplement upon the decolorization and degradation of Indigo dye containing denim industrial wastewater. All the analyses were conducted in triplicate and results presented here are the mean of triplicate ± standard deviations (± SE).